INVESTIGATIVE REPORT

Absence of an Active Metabolite for the Triazole Antifungal Pramiconazole

Jannie AUSMA[1], Gennethel PENNICK[2], Hilde BOHETS[3], Vera van de VELDE[4], Marcel BORGERS[1,5] and Annette FOTHERGILL[2]

[1]Barrier Therapeutics NV, Geel, Belgium, [2]Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA, [3]Johnson and Johnson Pharmaceutical Research and Development, a Division of Janssen Pharmaceutica NV, Beerse, Belgium, [4]Barrier Therapeutics Inc., Princeton, USA, and [5]Department of Molecular Cell Biology, University of Maastricht, Maastricht, The Netherlands

Pramiconazole is an antifungal with high potential for the treatment of dermatophyte and yeast infections of the skin. It is currently not known whether pramiconazole is active alone as the parent agent or assisted by active metabolites. The *in vitro* metabolism as well as the metabolic stability of pramiconazole was investigated in subcellular liver fractions and isolated hepatocytes of several species. Results indicate that the metabolism of pramiconazole was slow, since the enzyme-mediated disappearance of pramiconazole was rather slow. To investigate whether pramiconazole was converted into an active metabolite in humans, serum samples from healthy volunteers receiving a daily dose of 100 or 200 mg pramiconazole for one week were assayed with an agar diffusion bioassay and liquid chromatography-tandem mass spectrometry. It was concluded that there was no active metabolite present in serum samples from healthy volunteers after oral dosing of pramiconazole. *Key words: R126638; inhibition zone bioassay; metabolism; yeast; dermatophyte.*

Jannie Ausma, Barrier Therapeutics NV, Cipalstraat 3, B-2440 Geel, Belgium. E-mail: jausma@barriertherapeutics.be

Pramiconazole, previously referred to as R126638, is a broad-spectrum antifungal compound belonging to the class of the triazoles. It has excellent potential for oral and topical treatment of fungal infections of skin, hair, nails, oral and genital mucosa (1). There is evidence from *in vitro* data that pramiconazole is active against dermatophytes (*Trichophyton* spp., *Microsporum canis*, *Epidermophyton floccosum*) and yeasts (*Candida* spp. and *Malassezia* spp.). The high potency of pramiconazole is ascribed to its prominent affinity to fungal cytochrome P450, in particular 14α-demethylase, which is involved in the biosynthesis of ergosterol from lanosterol. Ergosterol is a vital component of cellular membranes in fungi, and its specific inhibition by pramiconazole results in inhibition of fungal growth (2). In mouse and guinea-pig models of dermatophyte and yeast infections a superior potency of pramiconazole over itraconazole was shown (1).

Isolated hepatocytes and subcellular liver fractions represent valid *in vitro* systems to study drug metabolism in different animal species (3).

Inhibition zone bioassays can be used for the determination of the antifungal activity of antifungals and their active metabolites in serum, as demonstrated for itraconazole (4–6). The metabolism of pramiconazole was investigated on subcellular liver fractions and isolated hepatocytes. To investigate whether or not the pramiconazole metabolites have antifungal activity, blood samples were collected from subjects who participated in a phase I trial (7). The antifungal concentration was determined for those subjects receiving a once-daily dose of either 100 or 200 mg for one week.

MATERIALS AND METHODS

In vitro study of the metabolism of pramiconazole

Metabolism experiments were performed in liver subcellular fractions (microsomes and $12,000 \times g$ fraction) of rat, mouse and human and in hepatocytes of rat, dog and human. Human liver pieces were obtained from kidney transplant donors (8).

In order to prepare liver subcellular fractions, livers were submerged in ice-cold 1.15% KCl – 0.01 M phosphate buffer pH 7.4 and minced with a pair of scissors. Crude homogenates were prepared in a cold room by homogenizing the liver pieces in isotonic phosphate buffer, using a Potter-Elvehjem homogenizer with rapidly rotating pestle (7 vertical strokes up and down) at a buffer/liver weight ratio of 3:1 (ml/g). 32 ml aliquots of crude homogenates were centrifuged in a Kontron TGA-50 ultracentrifuge equipped with a Kontron TFT 50.38 rotor or in a Beckman Optima XL-70 ultracentrifuge equipped with a Beckman 50-2 Ti rotor, at 12,000 rpm ($12,000 \times g$) for 20 min at 4°C. A part of the $12,000 \times g$ supernatant fractions were collected and frozen in small aliquots in glass tubes placed in liquid nitrogen. Microsomes were precipitated by centrifuging the $12,000 \times g$ supernatants at 36,000 rpm ($110,000 \times g$) for 1 h at 4°C. The microsomes were washed by resuspending the pellets in an equal volume of homogenization buffer, followed by centrifugation at $110,000 \times g$ for 1 h at 4°C. The final microsomal pellets were resuspended in 1.15% KCl – 0.01 M phosphate buffer pH 7.4. All microsomal fractions were characterized for protein- and cytochrome P-450 content. In addition, the metabolic capacity of each batch of human liver microsomes was checked by analysing the metabolic activity for different specific cytochrome P450-probe substrates. Protein concentrations were determined according to the method of Lowry et al. (9), as modified by Miller (10). The cytochrome P-450 content of fresh hepatocyte sus-

pensions or cell lysates was determined as described by Omura & Sato (11). The incubations of pramiconazole with subcellular liver fractions (both microsomes and 12,000 × g supernatant fractions) were performed at a protein concentration of 1 mg/ml in a total volume of 1.0 ml. The incubations with the 12,000 × g supernatants were performed at a protein concentration equivalent to that of the microsomes of the respective species. The co-factor mixture in each incubate contained 0.5 mg of glucose-6-phosphate, 0.25 units of glucose-6-phosphate dehydrogenase (only for incubations with microsomes), 0.125 mg of NADP and 0.5 mg of $MgCl_2.6H_2O$ in 0.5 ml of 0.5 M Na,K-phosphate buffer pH 7.4. After a pre-incubation of 5 min under continuous shaking at 100 oscillations/min, the reactions were started by adding 5-μl volumes of the pramiconazole stock solution (1.0 mM) to give a final concentration of 5 μM. The samples were incubated for 30, 60 and 120 min at 37°C under continuous shaking at 100 oscillations/min. The enzymatic reactions were stopped by freezing the samples in dry ice. The samples were stored at ≤ −18°C until analysis by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

Hepatocytes from rat and mouse were prepared in situ according to the two-step collagenase method, as described by Seglen (12). Dog and human hepatocytes were prepared according to the biopsy perfusion method (3, 13). Initial cell viability was determined by the trypan blue exclusion method. The metabolic capacity of the hepatocytes was determined using several cytochrome P-450 probe substrates. Metabolism experiments were performed with either suspension cultures or primary cell cultures. Primary cell cultures of hepatocytes were obtained by suspending the cells in RPMI 1640 medium supplemented with 10% foetal calf serum (Invitrogen corporation, Carlstad, California, USA), 1 μM insulin (from porcine pancreas, Sigma, St Louis, Missouri, USA), 2 mM L-glutamine (Gibco), 0.1 mM hydrocortisone 21-hemisuccinate (Sigma) and 1% penicillin-streptomycin solution (Gibco). The cells were seeded into 6-well multiplates (2 ml/well, $1.0 × 10^6$ cells/well). After at least 4 h, the culture medium containing non-adherent cells was removed and the cell layers were washed twice with RPMI 1640 medium. Incubations in suspension culture were performed at a concentration of 20 or 30 μM for 120 min. Incubations with hepatocytes in primary cell culture were performed at a concentration of 20 μM (dog and man) and at 30 μM (rat) for 21 h or 33 h, respectively.

Subject samples for the bioassay and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Plasma samples were obtained from the trial BT400BEL001 (7), in which healthy subjects received a 1-week of oral treatment with pramiconazole in oral solution: 8 subjects received 100 mg daily, 8 subjects received 200 mg daily and another 8 received placebo.

Venous blood samples of 5 ml were taken on day 1 at 4 h after drug intake and on day 7 just prior to intake of trial medication dose, and at 4 and at 24 h following drug intake. Blood samples were collected in heparinized tubes and centrifuged for 10 min at 1000 × g within 2 h of collection. Plasma was aspirated from the cells with a disposable pipette and transferred in 5 ml plastic tubes. The tubes were closed by means of polyethylene stoppers. The samples were stored at −20°C at C&T Paradigm before sending to the Fungus Testing Laboratory of the University of Texas Health Science Center, San Antonio, Texas, USA and the Analytisch Biochemisch Laboratorium (ABL), Assen, The Netherlands.

Bioassay method

The bioassay method is a previously described modification of a yeast nitrogen base agar diffusion method described by Bodet et al. (14). *Candida kefyr* (ATCC 46764) was added to a solution of yeast nitrogen base (YNB) broth and incubated at 37°C for 6 h. The *C. kefyr* solution was adjusted spectrophotometrically to a #2 McFarland standard and 0.5 ml was added to 45 ml of moultant YNB agar deeps. The YNB was poured into 150 × 15 mm Petri plates. A series of 7-mm wells were bored into the agar and filled with 50 μl of varying concentrations of pramiconazole standard (0.25, 0.5, 2, 5 and 10 μg/ml), assay controls (0.25, 1.0 and 8.0 μg/ml) or subject plasma. The plates were incubated overnight at 37°C. Antifungal activity is noted by zones of inhibition surrounding the specimen well. Zone diameters were measured in millimetres using a calliper and the standards' zone sizes vs. concentrations were plotted using Standard Curve Plus™ Software. For each standard curve, the equation chosen to determine the best-fit line fit type was y = a.ln(x) + b, where zone sizes (mm) were plotted on the y-axis and concentrations (μg/ml) on the log scale of the x-axis. The r^2 value for each curve was observed to be at least 0.99. Drug concentrations of the unknowns were determined from the standard curves by the software. The allowable coefficient of variation is approximately 15%.

The bioassay method for the determination of pramiconazole was validated using established guidelines to ensure acceptable performance of a microbiological method (15). The validation process allows determination of accuracy, precision, response function and reproducibility of the bioassay method for the determination of pramiconazole concentrations. The validation consisted of assaying 6 replicates of each control (0.25, 1.0 and 8.0 μg/ml) on 4 different run days. A standard curve consisting of 0.25, 0.5, 2.0, 5.0, 10.0 μg/ml was analysed with each batch of controls. The lowest limit of quantification was determined to be 0.25 μg/ml.

Spiked pramiconazole standards and controls used in the pramiconazole bioassay were sent to ABL, to be analysed by LC-MS/MS utilizing the same methods as used for the plasma samples. These results confirmed the validity of the standards used in the bioassay procedure.

Determination of the pramiconazole concentration in plasma by LC-MS/MS

Venous blood samples of 10 ml to obtain 5 ml of plasma were taken as described above.

At ABL, the concentrations of pramiconazole in plasma were determined by a LC-MS/MS method. This method was validated according to the FDA Guidance for Industry (16). The validated analytical range was 2.00–2000 ng/ml. In summary, the method was performed as follows. Under basic conditions, pramiconazole and the internal standard were extracted from plasma (100 μl) with tertiary butyl methyl ether isoamylalcohol (98:2). After evaporation of the extract and reconstitution in injection solvent, pramiconazole was quantified using a Sciex API4000 LC-MS/MS system.

Spiking of pramiconazole to plasma samples of study subjects

To investigate whether a direct or indirect alteration or interference in response due to unintended (for analysis) or other interfering substances in the sample (16) occurred in the bioassay, 6 plasma samples from the study subjects were spiked with different concentrations of pramiconazole. The samples were spiked with concentrations of 0.25, 0.5 and 1.0 μg/ml from pramiconazole stock solution (made from the stock 100 μg/ml in methanol). The samples were assayed by the above described bioassay method. Standard calibrators were made in pooled plasma from pramiconazole in methanol.

RESULTS

In vitro study on the metabolism of pramiconazole

Incubations with liver sub-cellular fractions, as well as with hepatocytes, demonstrated that the metabolism of pramiconazole was rather slow in all species, but was faster in rat and mouse than in human and dog. In human liver the maximum % metabolized protein after 120 minutes was 20.3% in the 12000 × g fraction and 7.5% in the microsomal fraction. Metabolites M1 and M2 could be identified in dog and rat based on retention times (Figs 1 and 2). Unidentified metabolites were observed in all species, based on retention times; no unique metabolites were observed in man (Fig. 1). Around 10% metabolites were found after about 30 h incubation in human hepatocytes in primary cell culture, similar results were obtained in rat and dog.

HPLC analysis with UV detection showed that the disappearance of pramiconazole was not reflected in the appearance of metabolites. By comparison with boiled samples it was shown that the disappearance of the pramiconazole was enzyme-mediated (Fig. 2).
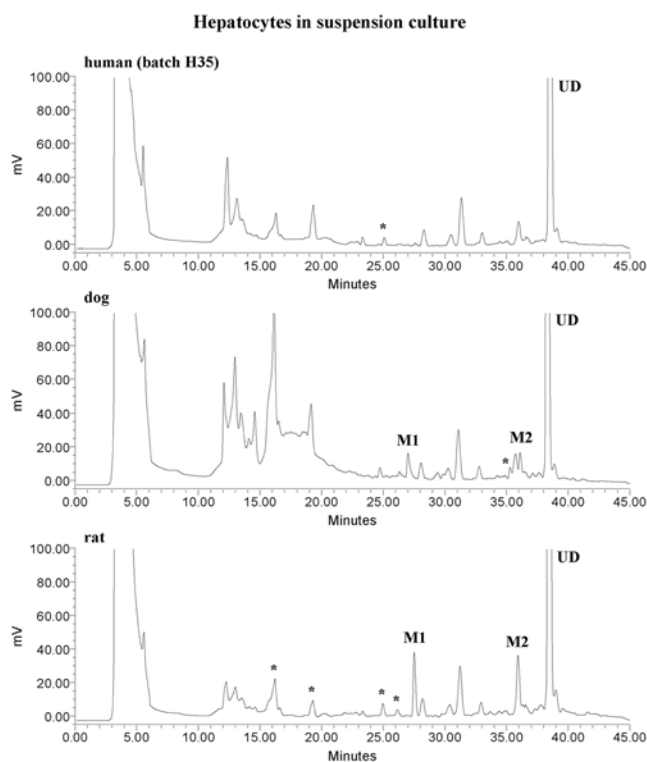

Fig. 1. Metabolism of pramiconazole in suspension cultures of hepatocytes of human, dog and rat. The high-performance liquid chromatography (HPLC) chromatograms were obtained for incubations of 20 μM (dog and human) and 30 μM (rat) pramiconazole. Metabolites were characterized by comparison of the ultra-violet (UV) spectrum of pramiconazole with the UV-spectrum of the metabolite and by comparison with the boiled microsomal fractions. UD represents the unchanged pramiconazole. Identified metabolites are denoted by M1 and M2, unknown metabolites by*.

Inhibition zone bioassay for assessment of the presence of active metabolites

Validation of the inhibition zone bioassay method. The validation consisted of assaying 6 replicates of each control (0.25, 1.0 and 8.0 μg/ml) on 4 different run days. A standard curve consisting of 0.25, 0.5, 2.0, 5.0 and 10.0 μg/ml working plasma standards was analysed with each batch of controls. The coefficient of variation ranged between 3.49 and 10.21%. For comparison of the controls between days, i.e. inter-day analysis, the overall imprecision (%CV) ranged from 9.9% to 10.8%, this is within the allowable coefficient of variation of 15%. Results of the validation predict that the pramiconazole bioassay method is reproducible, accurate and that the response factor of the calibration curve is linear according the algorithm $y = a. \ln(x) + b$, with R^2 ranging from 0.991 to 0.998.

Inhibition zone bioassay for subject samples from the study BT400BEL001

The selected samples from the subjects of the clinical trial BT400BEL001 were analysed by the bioassay. With each analysis run, the standard curve and controls were analysed. The concentrations measured with the inhibition zone bioassay were lower than those measured with the LC-MS/MS at all time points and for all samples analysed. In Fig. 3 individual data of the analysis are displayed. A similar concentration measured by the bioassay and the LC-MS/MS would be expected if an active metabolite was absent. Similarly, a higher
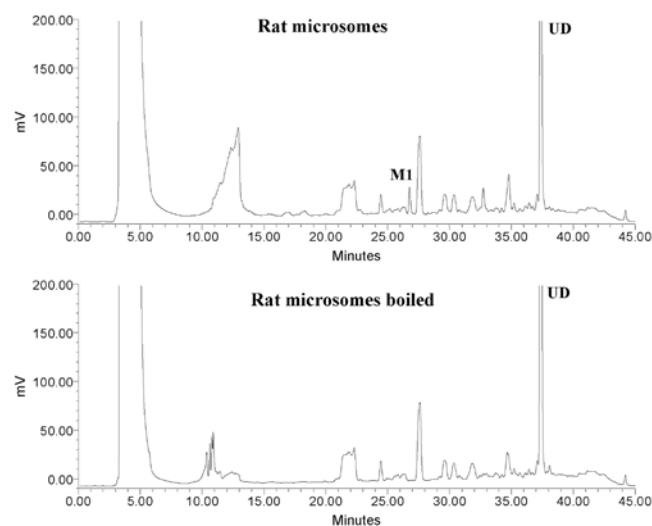

Fig. 2. Metabolism of pramiconazole in liver microsomes of rat. The high-performance liquid chromatography (HPLC) chromatograms were obtained for incubations of 10 μM. Metabolites were characterized by comparison of the ultra-violet (UV) spectrum of pramiconazole with the UV-spectrum of the metabolite and by comparison with the boiled microsomal fractions. UD represents the unchanged pramiconazole. Identified metabolites are denoted by M1.

concentration measured with the bioassay compared with LC-MS/MS would be expected if an active metabolite was present (4, 5). The presence of an active metabolite was not supported by the current results since the concentrations measured with the inhibition zone were lower than those measured with LC-MS/MS (Fig. 3). The slopes of the regression lines for the different sampling time points were not substantially different, which is supportive for the absence of an active metabolite. Assuming the absence of an active metabolite, the reduction of the slopes below 1 needs further explanation.

Spiking of pramiconazole to plasma samples of study subjects

A direct or indirect alteration or interference in response due to unintended or interfering substances in either the plasma samples from the study and those used for the standard curve, resulted in a lower detectable concentration of drug than is present in plasma and might therefore be held responsible for reduced concentration detection by the bioassay. To establish whether or not an alteration or interference is responsible for a lower apparent concentration of pramiconazole with the bioassay compared with the LC-MS/MS, 6 different plasma samples were spiked with 3 different concentrations of pramiconazole. In all samples the spiked pramiconazole could only be partially detected with the bioassay, where an average recovery of pramiconazole of $56.9 \pm 4.9\%$ (range 49.5–65.2%) was measured. The reduction seen by the bioassay could therefore be attributed to an interference or alteration. The results of the bioassay should therefore be corrected by 0.57. The regression lines at different time points after corrections have slopes ranging from 0.96 to 0.73. The current findings do not support the presence of an active metabolite

Fig. 3. Curves of the individual data points of the inhibition zone bioassay of samples taken at different time points together with the curve fits.
—＊— day 1, 4.00 h —△— day 7, 0.00 h
—◇— day 7, 4.00 h —⊖— day 7, 24.00 h
LC MS/MS: liquid chromatography-tandem mass spectrometry

after oral dosing of 100 or 200 mg pramiconazole once daily for one week.

DISCUSSION

Pramiconazole is a newly synthesized azole antifungal with high activity in animal models against dermatophyte and yeast infections (1). The inhibitory potential of pramiconazole on CYP 3A4, studied in liver microsomes, was much lower than that of itraconazole (2).

The enzyme-mediated metabolism of pramiconazole in subcellular liver fractions as well as hepatocytes was slow. Pramiconazole showed a high metabolic stability in hepatocytes, especially compared with itraconazole, which was shown to be extensively metabolized (Karel Lavrijsen J&J PRD, personal communication). Ketoconazole was converted into many metabolites as became clear from *in vivo* studies (17). Fluconazole, however, is metabolically stable since high excretion levels of unchanged drug were found *in vivo* (18).

It might be that the slow metabolism of pramiconazole can be explained by the formation of metabolites that affect the metabolic turnover of pramiconazole, a phenomenon described for other azoles like itraconazole (19). Pharmacological studies of itraconazole indicated differences between bioassay and chromatographic determinations due to the presence of an active metabolite.

A simple agar-well diffusion bioassay was previously developed for measurement of antifungal concentrations using a *C. kefyr* strain. A linear relationship between zone diameters and log10 concentrations of pramiconazole, as for ketoconazole or flucytosine, was observed (14). For pramiconazole, the coefficient of variation was within the allowable variation and comparable with described coefficients of variation for itraconazole. Although the agar diffusion bioassay method has a rather high variation, the method gives a good indication for the presence of an active metabolite for an antifungal. In case of absence of an active metabolite, extensive studies with metabolite identification may be avoided. For pramiconazole, further investigational studies for identification of metabolites with antifungal activity may not be needed, since (*i*) lower concentrations were measured with the bioassay compared with the LC-MS/MS at all time points and for all subjects analysed and (*ii*) the slopes of the regression lines for different sampling time points were not substantially different.

In contrast, itraconazole concentrations measured by bioassay indicated antifungal activity in the samples at a factor of 2 times higher than the total azole level determined (5, 6, 20).

The reduction of all slopes below 1, as seen in our study, needs further clarification. The mechanism and origin of the alterations or interferences in plasma samples are not well understood, but might originate from interactions between an analyte and the sample
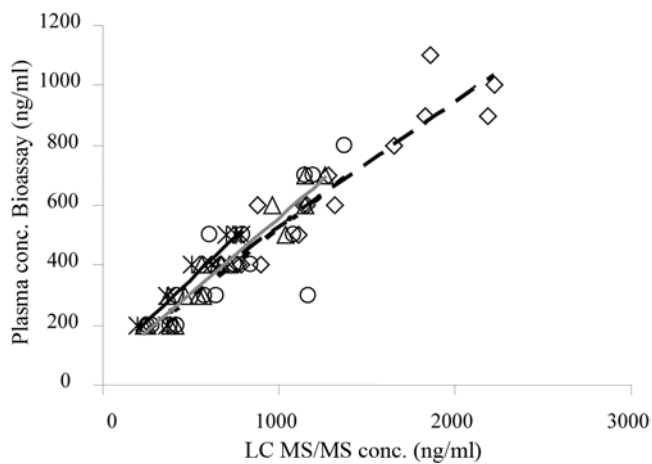
preparation, including components in serum samples or undetectable matrix components in the reaction environment (agar diffusion plates used for the bioassay inhibition zone test). Cross-validation of the bioassay with spiked pramiconazole standards in subject samples was carried out to determine the extent of alterations or interferences.

The spiked pramiconazole could only be partially detected with the bioassay, after correction of all plasma samples the regression lines at different time points have slopes within the acceptable range.

In conclusion, pramiconazole is a drug of promise for development as a new triazole for dermatological use with a slow enzyme-mediated metabolism. The presented findings suggest that pramiconazole is not converted into an active metabolite after oral dosing in humans of 100 or 200 mg daily for one week.

REFERENCES

1. Odds F, Ausma J, Van Gerven F, Woestenborghs, Meerpoel L, Heerens J, et al. Activity *in vitro* and *in vivo* of a novel azole antifungal agent R126638 Antimicrob Agents Chemother 2004; 43: 388–391.
2. Vanden Bossche H, Ausma J, Bohets H, Vermuyten K, Willemsens G, Marichal P, et al. The novel azole R126638 is a selective inhibitor of ergosterol synthesis in *Candida albicans, Trichophyton* spp. and *Microsporum canis*. Antimicrob Agents Chemother 2004; 48: 3272–3278.
3. Moshage H, Yap SH. Primary cultures of human hepatocytes: a unique system for studies in toxicology, virology, parasitology and liver pathophysiology in man. J Hepatol 1992; 15: 404–413.
4. Hostetler JS, Heykants J, Clemons KV, Woestenborghs R, Hanson LH, Stevens DA. Discrepancies in bioassay and chromatography determinations explained by metabolism of itraconazole to hydroxyitraconazole: studies of interpatient varieties in concentrations. Antimicrob Agents Chemother 1993; 37: 2224–2227.
5. Hülsewede JW, Dermmouni H, Ansorg R. Determination of itraconazole and hydroxy-itraconazole in sera using high-performance liquid chromatography and a bioassay. Zentralbl Bakteriol 1995; 282: 457–464.
6. Odds FC, Dupont B, Rinaldi MG, Stevens DA, Warnock DW, Woestenborghs R. Bioassay for itraconazole blood levels: an interlaboratory collaborative study. Antimicrob Chemother 1999; 43: 723–727.
7. Ausma J, Bruynseels J, Snoeck E, Blockhuys S, van Rossem K, Borgers M. Placebo-controlled randomized double-blind multiple dose dose-rising trial in healthy volunteers to assess the tolerability, safety and pharmacokinetics of the novel antifungal R126638. J Eur Acad Derm Venereol 2004; 18 (suppl. 2): 392.
8. Bohets H, Lavrijsen K, Hendrickx J, Van Houdt J, van Genechten V, Verboven P, et al. Identification of the cytochrome P450 enzymes involved in the metabolism of cisapride: in vitro studies of potential co-medication interactions. Br J Pharmacol 2000; 129: 1655–1667.
9. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenon reagent. J Biol Chem 1951; 193: 265–275.
10. Miller GL. Protein determination for large number samples. Anal Chem 1959; 31: 964.
11. Omura T, Sato R. The carbon monoxide pigment of liver microsomes. II. Solubilization, purification and properties. J Biol Chem 1964; 239: 2379–2385.
12. Seglen PO. Preparation of rat liver cells. I. Effect of Ca^{2+} on enzymatic dispersion of isolated perfused liver. Exp Cell Res 1972; 74: 450–454.
13. Reese JA, Byard JL. Isolation and culture of adult hepatocytes from liver biopsies. In Vitro 1981; 17: 935–940.
14. Bodet CA, Jorgensen JH, Drutz DJ. Simplified bioassay method for measurement of flucytosine or ketoconazole. J Clin Microbiol 1985; 22: 157–160.
15. Shah V, Midha K, Dighe S, McGilveray I, Skelly J, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence, and pharmacokinetic studies. J Pharmaceutical Sci 1992; 81: 309–312.
16. Guidance for Industry. Bioanalytical method validation. US Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001.
17. Gascoigne EW, Barton GJ, Michaels M, Meuldermans W, Heykants J. The kinetics of ketoconazole in animals and man. Clin Res Rev 1981; 1: 177–187.
18. Feczko J: Fluconazole: an overview; In: Fluconazole and its role in vaginal candidiasis. Richardson RG, ed. International Congress and Symposium Series No. 160. London: Royal Society of Medicine Services, 1989: p. 11–18.
19. Isoherranen N, Kunze KL, Allen KE, Nelson WL, Thummel KE. Role of itraconazole metabolites in CYP3A4 inhibition. Drug Metab Dispos. 2004; 32: 1121–1131.
20. Heykants J, Van Peer A, Van de Velde V, Van Rooy P, Meuldermans W, Lavrijsen K, et al. The clinical pharmacokinetics of itraconazole: an overview. Mycosis 1989; 32 (suppl 1): 67–87.